ISSN 1346-7565 Acta Phytotax. Geobot. 59 (2): 165–174 (2008)

Origin of the Endangered Tetraploid *Adonis ramosa* (Ranunculaceae) Assessed with Chloroplast and Nuclear DNA Sequence Data

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Polyploidization is an important evolutionary force in plant speciation, and elucidating the origin and evolutionary history of a particular polyploid is important for understanding the evolution and distribution of plants. We investigated the origin of the endangered tetraploid *Adonis ramosa* Franch., which is endemic to Japan. To clarify the origin and phylogenetic relationships among *A. ramosa* and diploid species, we conducted a phylogenetic analysis of the species of *Adonis* in Japan and Korea using chloroplast *trnL–trnF* spacer and nuclear ribosomal internal transcribed spacer (ITS) sequences. The sequences of the ITS region of showed that the sequences of *A. ramosa* were completely consistent with or quite similar to those of *A. amurensis* Regel & Radde of Hokkaido, Japan, and clearly differed from those of *A. amurensis* in Korea and three diploid species from Japan and Korea. The results suggest the possibility that *A. ramosa* is an autotetraploid, and the diploid progenitor is *A. amurensis* from Hokkaido. The distribution pattern of the ITS haplotype of *A. ramosa* also indicates range expansion of *A. ramosa* from northern to southern Japan Ecological divergence and adaptation to new habitats after polyploidization are likely to increase the survival of *A. ramosa* and enable it to widely colonize in new environments in Japan.

Key words: *Adonis ramosa*, ecological divergence, Far East Asia, internal transcribed spacer (ITS), polyploidy, *trnL–trnF* spacer

Polyploidization is an important evolutionary force in plant speciation (Soltis & Soltis 1999). Polyploids often differ from their diploid progenitors in morphological and ecological aspects, and neopolyploids may replace their diploid progenitors and colonize ecological niches unsuitable for their diploid progenitors because of superior fitness (Levin 2002). Therefore, elucidating the origin and evolutionary history of a particular polyploid is important for understanding the evolution and distribution of the species.

Adonis ramosa Franch. is a tetraploid ($2n$ = 32) endemic to Japan. It is distributed across the islands of Hokkaido, Honshu, and Shikoku (Fig. 1) and is the most common species of *Adonis* in Japan. Although *A. ramosa* was believed to

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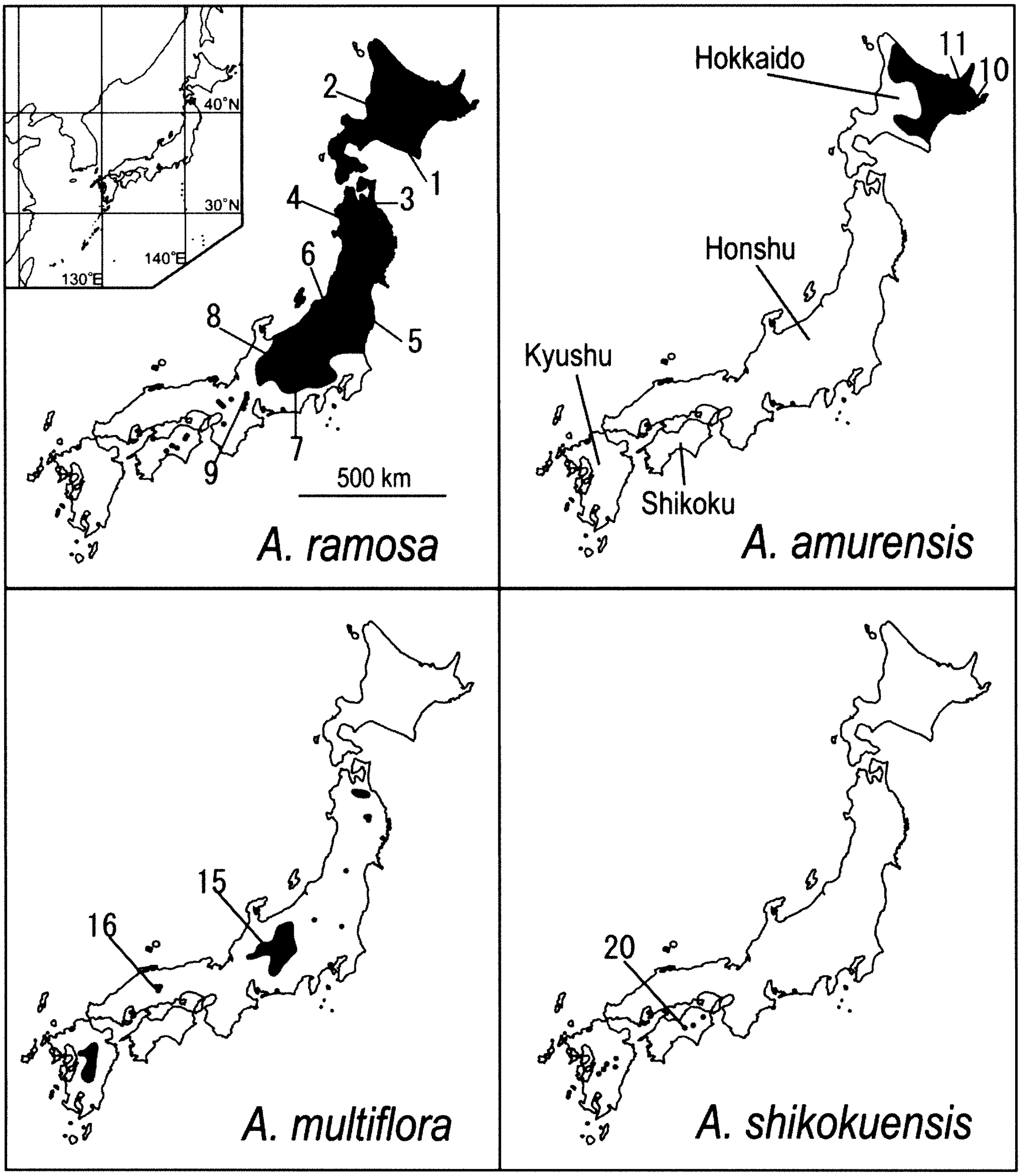

FIG. 1. Distribution of four species of *Adonis* in Japan (modified from Kawano & Hayashi 2004). The numbers indicate sampling locations shown in Table 1.

be the only species of *Adonis* native to Japan (Satake *et al.* 1982, Ohwi & Kitagawa 1983), recent morphological studies and chromosome analysis (Nishikawa 1988, 1989a, b, Nishikawa & Ito 2001) have shown that *Adonis* in Japan consists of four species: *A. ramosa* Franch., *A. amurensis* Regel & Radde, *A. multiflora* Nishikawa & Koji Ito (Nishikawa 1989a), and *A. shikokuensis* Nishikawa & Koji Ito (Nishikawa & Ito 2001). *Adonis amurensis* is found in northern and eastern Hokkaido, and is widely distributed around the Japanese Archipelago, for example in Far East Russia, including Sakhalin and the Kurile islands, northeastern China, and the Korean pen-

insula (Hoffmann 1998). *Adonis multiflora* occurs on Honshu and Kyushu in Japan and on Cheju Island in Korea (Kawano & Hayashi 2004, Suh *et al.* 2002). *Adonis shikokuensis* has been reported from Shikoku and Kyushu in Japan (Nishikawa & Ito 2001).

Four of five species of *Adonis* in Far East Asia, including *A. amurensis, A. multiflora, A. shikokuensis,* and *A. pseudoamurensis* Wang (Wang 1980) from China and Korea, are diploid ($2n$ = 16; Nishikawa 1988, Ahn *et al.* 1999, Nishikawa & Ito 2001); *Adonis ramosa* is tetraploid ($2n$ = 32). *Adonis ramosa* is therefore likely to have originated from one or two of the diploid species. Even though *A. ramosa* has been known for several decades to be a tetraploid (Nishikawa & Ito 1978), little information about the origin of *A. ramosa* has been reported, and there has been insufficient evidence to pinpoint its origin. In this report, we present information on the origin of *A. ramosa* using the *trnL–trnF* chloroplast spacer and the internal transcribed spacer (ITS) of the nuclear DNA sequence data of *Adonis* in Japan, and publish ITS sequence data for the species of *Adonis* in Korea.

Materials and Methods

We collected leaf samples from 14 populations of four species of *Adonis* in Japan (Table 1). Plant materials were kept fresh on ice after collection in the field. The samples were frozen to –30°C in the laboratory until DNA extraction. Genomic DNA was extracted from leaves using a modified CTAB method (Milligan 1992).

The non-coding regions of the chloroplast DNA between the *trnL*(UAA) 3′exon and the *trnF* (GAA) (Taberlet *et al.* 1991) and the internal transcribed spacer (ITS) of nuclear ribosomal DNA between ITS5 and ITS4 (White *et al.* 1990) were amplified by PCR and then sequenced. We amplified the ITS and *trnL–trnF* regions with AmpliTaq Gold (Applied Biosystems, Foster City, California, USA). The PCR amplification was performed in a thermal cycler (GeneAmp PCR System 2700, Applied Biosystems) under the following conditions: for the *trnL–trnF* region, initial denaturation at 94°C for 9 min was followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 2 min, and final extension at 72°C for 7 min; and for the ITS region, initial denaturation at 94°C for 9 min was followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min, and final extension at 72°C for 7 min. The PCR products were purified using a High Pure PCR Product Purification Kit (Roche Applied Science, Penzberg, Germany). The purified products were sequenced directly with an ABI BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems) on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Electropherograms were assembled with Sequencher 3.1 software (GeneCodes, Ann Arbor, Michigan, USA), and sequences were aligned using CLUSTAL X (Thompson *et al.* 1997).

We determined the *trnL–trnF* and ITS haplotypes based on site change data and indels. In the *trnL–trnF* region, we compared results between haplotypes of *Adonis ramosa* and three other species of *Adonis* (*A. amurensis*, *A. multiflora*, and *A. shikokuensis*) from Japan. For the ITS region, the sequence data of three species of *Adonis* (*A. amurensis*, *A. multiflora*, and *A. pseudoamurensis* Wang) from Korea and *A. vernalis* L. from Hungary (DDBJ DNA data base accession numbers AF454924 to AF454936, Suh *et al.* 2002) were also used for analysis. Based on the sequence data, phylogenetic analyses were performed using maximum parsimony methods. The most parsimonious trees were obtained with PAUP 4.0 (Swofford 2001) using heuristic searches with 100 random taxon addition replicates and TBR swapping. Gaps were excluded and site changes

TABLE 1. ITS and cpDNA variation of *Adonis* in Japan and Korea.

Species, No. and locality		Haplotype[a] *trnL–F*	Haplotype[a] ITS	Accession No. *trnL–F*	Accession No. ITS
Adonis ramosa					
1	Urakawa, Hokkaido Prefecture, Japan	A	A	AB361596	AB361610
2	Furano, Hokkaido Prefecture, Japan	A	A	AB361597	AB361611
3	Hachinohe, Aomori Prefecture, Japan	A	A	AB361598	AB361612
4	Fujisato, Akita Prefecture, Japan	A	A	AB361599	AB361613
5	Yonezawa, Yamagata Prefecture, Japan	A	A	AB361600	AB361614
6	Tadami, Fukushima Prefecture, Japan	A	A	AB361601	AB361615
7	Ina, Nagano Prefecture, Japan	A	B (1ID)	AB361602	AB361616
8	Asahi, Gifu Prefecture, Japan	A	B (1ID)	AB361603	AB361617
9	Mt. Fujiwara, Mie Prefecture, Japan	A	B (1ID)	AB361604	AB361618
Adonis amurensis					
10	Nemuro, Hokkaido Prefecture, Japan	A	A	AB361605	AB361619
11	Shari, Hokkaido Prefecture, Japan	A	A	AB361606	AB361620
12	Mt. Jeoksang, Chonbuk Province, Korea	—	C (10NS, 2ID)	—	AF454927
13	Mt. Chookryung, Kyunggi Province, Korea	—	D (10NS)	—	AF454928
14	Daekwanryuung , Kyungwon Province, Korea	—	C (10NS, 2ID)	—	AF454929
Adonis multiflora					
15	Kawai, Gifu Prefecture, Japan	B (2NS)	I (15NS, 1ID)	AB361607	AB361621
16	Shoubara, Hiroshima Prefecture, Japan	B (2NS)	I (15NS, 1ID)	AB361608	AB361622
17	Mt. Halla, Cheju Island, Korea	—	J (15NS)	—	AF454924
18	Mt. Halla, Cheju Island, Korea	—	E (14NS)	—	AF454925
19	Sangumbri, Cheju Island, Korea	—	E (14NS)	—	AF454926
Adonis shikokuensis					
20	Ootoyo, Kouchi Prefecture, Japan	B (2NS)	E (14NS)	AB361609	AB361623
Adonis pseudoamurensis					
21	Euyung, Kyungam Province, Korea	—	F (15NS)	—	AF454930
22	Kwanchon, Chunbuk Province, Korea	—	E (14NS)	—	AF454931
23	Mt. Palgong, Kyungbuk Province, Korea	—	E (14NS)	—	AF454932
24	Mt. Kyeryong, Choongnam Province, Korea	—	G (15NS)	—	AF454933
25	Chollipo, Choongnam Province, Korea	—	G (15NS)	—	AF454934
26	Jangbong Island, Kyunggi Province, Korea	—	H (14NS)	—	AF454935
Adonis vernalis (outgroup)					
27	Mt. Cserhat, Hungary	—	Out (20NS)	—	AF454936

[a] The number of variable sites compared to haplotype A is shown in parentheses; NS, Nucleotide substitution; ID, Insertion/Deletion. A dash (—) indicates that the nucleotide sequence of the region has not determined.

were weighted equally in the analysis. The phylogenetic tree was rooted by the outgroup comparison. A strict consensus tree was constructed from the most parsimonious trees, and bootstrap values were estimated with 1,000 replicates using the heuristic search option.

Results

In the *trnL–trnF* region (299 bp), sequences of *Adonis ramosa* were completely consistent with those of *A. amurensis* in Japan, but differed from *A. multiflora* and *A. shikokuensis* (Table 1). In the ITS region (603bp), we detected 36 variable

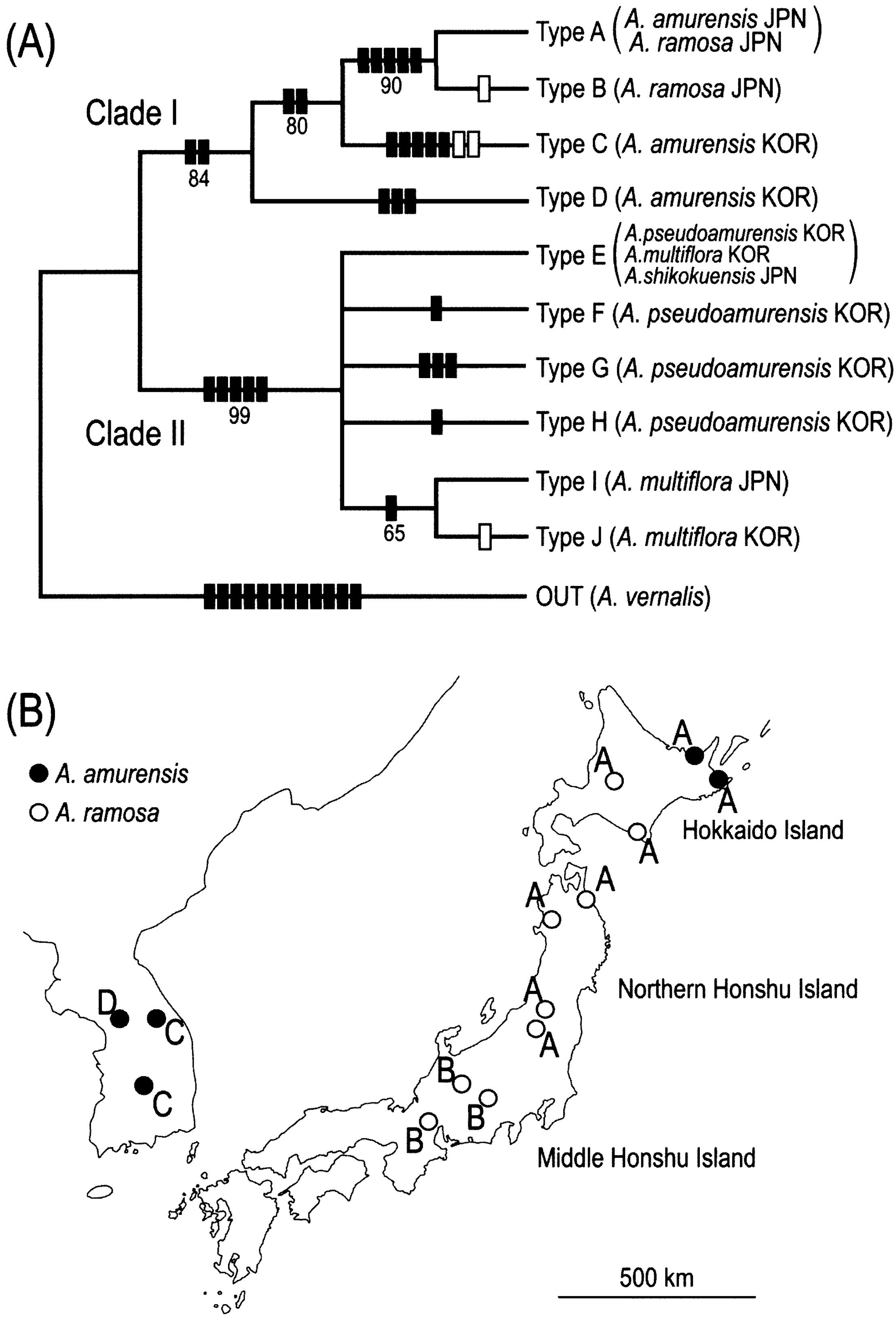

FIG. 2. (A) The strict consensus tree of the four most parsimonious trees among the ITS haplotypes of species of *Adonis* using site changes. Numbers below branches are bootstrap values in percentages based on 1,000 replicates. Solid and open bars represent site changes and indels, respectively. (B) Distribution of haplotypes comprising Clade I. Letters indicate haplotypes of *A. amurensis* (solid circle) and *A. ramosa* (open circle).

sites including 17 phylogenetically informative sites. In the phylogeny of ten haplotypes based on ITS sequences of five species of *Adonis*, we identified two distinct clades (Fig. 2A). Clade I was composed of *A. amurensis* and *A. ramosa*; Clade II was composed of *A. multiflora, A. shikokuensis,* and *A. pseudoamurensis*. ITS Sequences for the nine populations of *A. ramosa* were completely consistent or quite similar to those of *A. amurensis* in Japan, but clearly differed from those of *A. amurensis* in Korea (Table 1, Fig. 2A). In addition, intraindividual single-site polymorphisms as overlapping double peaks were not detected in the ITS region for all *A. ramosa* samples. *Adonis ramosa* from Hokkaido and northern Honshu (Nos. 1–6) showed identical sequences with those of *A. amurensis* from Hokkaido (Type A). *A. ramosa* from central Honshu (Nos. 7–9) showed a derivative and specific haplotype (Type B), which was distinguished from Type A haplotype by one indel (Table 1, Fig. 2B) These results showed that *A. amurensis* is phylogenetically the closest diploid species to *A. ramosa*, and furthermore, that *A. amurensis* from Hokkaido is closer to *A. ramosa* than to *A. amurensis* from Korea with haplotypes C and D.

Discussion

In the *trnL–trnF* region of the chloroplast DNA, the sequences of *Adonis ramosa* were completely consistent with those of *A. amurensis* in Japan, but differed from *A. multiflora* and *A. shikokuensis,* indicating that the maternal parent (cytoplasmic donor) of *A. ramosa* is *A. amurensis.* In the ITS region of the nuclear DNA, sequences of *A. ramosa* were identical or quite similar to those of *A. amurensis* in Japan. In the ITS region, examples of concerted evolution which has completely erased traces of other possible parents were reported (Wendel *et al.* 1995, Truyens *et al.* 2005). It is therefore undesirable to determine polyploid origin based only on ITS sequence data. ITS sequence analysis in many allopolyploid taxa, however, have shown that both parental ITS sequence or chimeric (mosaic like) ITS repeat types of parental motifs as overlapping double peaks (Kim & Jansen 1994, O'Kane *et al.* 1996, Franzke & Mummenhoff 1999, Vargas *et al.* 1999, Gaut *et al.* 2000, Hughes *et al.* 2002, Wichman *et al.* 2002, Koch *et al.* 2003, Lihova *et al.* 2004, Marhold *et al.* 2004, Rauscher *et al.* 2004, Devos *et al.* 2006). The lack of intraindividual polymorphisms in the *A. ramosa* sequences therefore suggests the possibility that *A. ramosa* is an autotetraploid, and that the diploid progenitor is *A. amurensis* from Hokkaido.

Autopolyploid plants were once considered extremely rare in nature. They usually reproduce asexually, either by vegetative propagation or apomixis, because of difficulties in meiosis (Futuyma 1986). Recent studies from various angiosperm lineages, however, have indicated that autopolyploidy is much more common than once considered (Soltis *et al.* 2004), and sexual autotetraploidy has been reported in several taxa, including *Chamerion angustifolium* (reported as *Epilobium angustifolium*; Onagraceae; Husband & Schemske 1997), *Aster kantoensis* (Asteraceae; Inoue *et al.* 1998), *Ranunculus cassubicifolius* (Ranunculaceae; Hörandl & Greilhuber 2002), and *Campanula americana* (Campanulaceae; Galloway *et al.* 2003). *Adonis ramosa* produces fertile seeds. Hand-pollination experiments have shown that seed-set is increased by outcrossing (Kudo 1995). These observations indicate sexual reproduction in *A. ramosa,* which is likely to be a sexual autotetraploid.

ITS sequences of *Adonis amurensis* in Japan and Korea showed high intraspecific variability. The ITS sequences of *A. amurensis* from Hokkaido were identical or quite similar to those of *A. ramosa*, suggesting that *A. ramosa* was derived from *A. amurensis*, now restricted to Hokkaido,

by autopolyploidization. The high similarity of the ITS sequences among *A. ramosa* and *A. amurensis* in Japan suggests that speciation occurred recently. Although intraspecific variability in *A. ramosa* was low and showed only two ITS haplotypes, distribution patterns of the haplotype are likely to indicate range expansion after polyploidization. The ITS sequences of *A. ramosa* from Hokkaido and northern Honshu were consistent with those of *A. amurensis* in Hokkaido (Type A), but *A. ramosa* from central Honshu showed a derivative and specific haplotype of *A. ramosa* (Type B; Fig. 2B). It is therefore likely that the range expansion of *A. ramosa* was as follows. *Adonis ramosa* with the Type A haplotype colonized southern Hokkaido and northern Honshu first, followed by a one indel mutation in the process of range expansion into northern and central Honshu. *Adonis ramosa* with the Type B haplotype then spread in central Honshu.

Tetraploid *Adonis ramosa* clearly ranges more widely than does its diploid progenitor *A. amurensis* in Japan (Fig.1). Like many allotetraploids, autopolyploids are not always more vigorous than their progenitors (Rieseberg & Ellstrand 1993, Rieseberg 1995, Soltis & Soltis 1995, Song *et al.* 1995). However, combined analysis of ecological niche modelling and molecular phylogeography for autotetraploid and diploid *Hordeum gussoneanum* (Poaceae) shows that autopolyploids dominate ecological niches that are unsuitable for their diploid progenitors (Jakob *et al.* 2007). A similar difference in distribution among autopolyploids and diploid progenitors was found in autotetraploids and diploids of *Biscutella laevigata* (Brassicaceae) in the western Alps (Tremetsberger 2002), and the autotetraploid *Taraxacum venustum* (Asteraceae) and diploid *T. platycarpum* subsp. *hondoense* in Far East Asia (Morita 1976, Akhter *et al.* 1993).

These differences in distribution between polyploids and their diploid progenitors may be the result of ecological divergence after polyploidization. For example, ecological differences between *Adonis ramosa* and *A. amurensis* are evident in their seed production and habitats. Because of the larger number of flowers on *A. ramosa* compared to *A. amurensis* (Nishikawa & Ito 2001), seed production in *A. ramosa* is greater than in *A. amurensis* (Nishikawa 1988, Kaneko *et al.* 2005). With regard to habitats, *A. amurensis* grows on grassy slopes and in broadleaved deciduous forests (Nishikawa & Kadota 2006, Kaneko pers. obs.). *Adonis ramosa* also occurs in these habitats on Hokkaido, but on Honshu the characteristic habitats of *A. ramosa* are grassy banks of terrace fields, meadows, and clearcut slopes around houses. Populations occurring in broadleaved deciduous forests are uncommon (Kaneko pers. obs.). It appears that adaptation to environments created by humans is a distinct feature of *A. ramosa*.

The human impact of grassland management in rural environments affects the survival of *Adonis ramosa*, especially on Honshu. On Honshu, grassland management has been conducted for at least 10,000 years (Yamanoi 1996, Ogura 2002), and grasslands managed by humans are likely to provide habitat for *A. ramosa*. Changes in methods of agricultural, however, have caused a massive reduction in traditionally managed grasslands since the 1960s. In recent years, populations of *A. ramosa* have declined through habitat loss because of the abandonment of traditionally managed grasslands. *Adonis ramosa* is now classified as "vulnerable" in the Japanese Red Data Book (Environment Agency of Japan 2000).

Our analysis using molecular markers shows that *Adonis ramosa* is derived from one lineage of *A. amurensis*, now distributed on Hokkaido, by autopolyploidization. Ecological divergence and adaptation to new habitats after polyploidization likely increased the survival of *A. ramosa* in Japan and enabled it to colonize rural environ-

ments. Rapid environmental change and habitat alteration in recent years may have endangered *A. ramosa*. The present findings are important for understanding the evolutionary history of *Adonis*, and provide basic knowledge on long-term ecological conservation of *A. ramosa*.

We thank S. Goto, H. Ida, K. Ishida, and A. Uchida for their support of this research. This study was partly supported by a Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Agriculture, Forestry and Fisheries of Japan, and the 21st Century Center of Excellence Program at Hiroshima University.

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Received November 7, 2007; accepted May 31, 2008